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European Food Research and Technology

ISSN 1438-2377

Eur Food Res Technol
DOI 10.1007/s00217-019-03305-0

Springer

European Food Research and Technology
https://doi.org/10.1007/s00217-019-03305-0

ORIGINAL PAPER

Structure and in vitro digestibility of grass pea (*Lathyrus sativus* L.) flour following transglutaminase treatment

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Received: 21 January 2019 / Revised: 13 May 2019 / Accepted: 26 May 2019

Abstract

The impact of transglutaminase (TG) modification on microstructure and in vitro protein and starch digestibility of grass pea flour was investigated. Results demonstrated that grass pea flour proteins act as effective substrate of TG. Microstructural results showed that the addition of TG produced a more compact structure likely due to TG-catalyzed heteropolymers. Nutritional properties such as slowly digestible starch and expected glycemic index values followed the order: grass pea flour incubated in the absence of TG>grass pea flour incubated in the presence of TG>raw flour. The TG-catalyzed heteropolymers were easily digested as demonstrated by in vitro oral and gastric digestion carried out under physiological conditions. Therefore, TG-modified grass pea flour can be considered as a new source of starch and proteins suitable for feeding a large spectrum of population.

Keywords Grass pea flour · In vitro digestion · Food structure · Transglutaminase · Estimated glycemic index

Introduction

Grass pea (*Lathyrus sativus* L.) is a very popular crop in many Asian and African countries where it is grown either for stockfeed or human consumption [1]. It is characterized by a lot of advantageous biological as well as agronomic features such as resistance to drought, high grain-yielding capacity and high protein content of its seeds [2]. There is a great potential for the expansion in the utilization of grass pea in dry areas and zones which are becoming more drought prone as a result of climate change [3]. Grass pea belongs to the leguminous family and is high in protein (28.70 g/100 g) and lysine contents [3]. However, the grass pea seeds, only when eaten as a large part of the diet for long time, can cause lathyrism [4] due to the presence of a non-protein aminoacid β-*N*-oxalyl-L-α, β-diaminopropionic acid (β-ODAP). When grass pea is a part of a varied diet, β-ODAP is tolerated without any known adverse effect. Thus, nowadays, this legume is rightly considered as one of the most promising sources of starch and proteins [5].The use of pulses is bound to increase in the future, and especially in combination with cereal raw materials they may find new applications, meeting both sensory and nutritional needs of consumers worldwide. However, it is necessary that different kinds of crops are studied to obtain structural parameters and information required to gain competitiveness in an international-scale industry. Pulses have recently gained interest as protein sources because of their high-quality protein (about 20–40%) [6], nutrient density [7] and as suitable ingredients in gluten-free foods. It has long been established that pulses are low glycemic index [8, 9] and there is growing evidence that eating pulse foods regularly reduces serum cholesterol [10]. Additional health benefits of pulses have been revealed through recent research [11].

As a follow-up to a former study about properties and in vitro digestion of raw Grass pea (*Lathyrus sativus*) flour [12, 13], one of the aims of the present research was to study the proteins of grass pea flour as microbial transglutaminase (TG) substrate. TG catalyzes intra- and/or intermolecular isopeptide bonds between the γ-carboxamide group of

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Published online: 03 June 2019

glutamine (acyl donor) and ε-amino group of lysine residues (acyl acceptor) isopeptide bonds between glutamines and lysines into proteins [14–17]. Hence, the impact of the TG modification on microstructure, in vitro protein and starch digestibility as well as on the expected glycemic index (GI) of grass pea flour was explored.

Materials and methods

Seed materials

Grass pea seeds (*Lathyrus sativus*) were purchased by "La Bona Usanza (S.C.A.R.L.) as Slow Food Presidia [18]. Plants (Population C3, characterized for the ODAP content [19] were grown in the field in Serra de' Conti Municipality, Ancona Province (central Italy) in the summer 2016.

Reagents

TG, Activa TI (specific activity 92 U/g), Ajinomoto, Japan, was provided by Prodotti Gianni Reagents. Gels for SDS-PAGE were from Bio-Rad (Segrate, Milano, Italy). α-amylase (product A1031), pepsin from porcine gastric mucosa (product P6887) and all other reagents were purchased from Sigma Chemical Company (Pool, Dorset, UK). Chemicals were of analytical grade, unless specified.

Flour preparation

Grass pea flour was prepared according to Romano et al. [13] and Al-Asmar et al. [20] by grounding the grass pea seeds using a variable speed laboratory blender (LB20ES, Waring Commercial, Torrington, Connecticut, USA), so that the grass pea flour (raw) would pass through a 425-µm stainless steel sieve (Octagon Digital Endecotts Limited, Lombard Road, London, UK).

Some flour samples were boiled in water for 15 min to simulate the cooking process. Moreover, cooked samples were treated for 2 h at 37 °C in the absence (GP) and presence of TG (20 U/gr of substrate) (GP/TG) as described in the next paragraph. All grass pea flour samples (raw, GP and GP/TG) were collected and stored in polyethylene bags at 4 °C until used for analysis.

TG-mediated modification of grass pea flour

The enzymatic modification of raw flour by means of TG was carried out by following the procedure described in Mariniello et al. [14] and Porta et al. [21] with some modifications. It is worth to note that the samples were treated for 15 min at 100 °C to allow protein denaturation and in the same time to simulate the cooking process. Briefly, 100 µg of protein flour were incubated in Tris–HCl 80 mM pH 7.5 with increasing (5, 10, 20 U/g) amounts of TG for 2 h at 37 °C in a final volume of 100 µL. The same experiment was carried out also on unheated flour with the aim of comparing the extent of TG-catalyzed reaction following the denaturation process.

Microstructural analysis

All flour samples (raw, GP and GP/TG) were dried at the critical point and coated with gold particles in an automated critical point dryer (model SCD 050, Leica Vienna). Microstructure of samples was examined by means of Scanning Electron Microscopy (SEM) (LEO EVO 40, Zeiss, Germany) as reported by Romano et al. [13] at a magnification of ×2000.

In vitro starch digestibility and expected glycemic index

Measurement of resistant starch (RS) and non-resistant starch (solubilised, Non-RS) were determined using an enzymatic assay kit (Resistant Starch Assay Kit, Megazyme International, Ireland) by AACC [22]. Starch hydrolysis is expressed as the ratio of Non-RS to the sum of RS and Non-RS starch [23]. All these results were expressed as percentage weight/weight on dry basis.

Rapidly digestible starch (RDS) and slowly digestible starch (SDS) were measured after incubation for 30 min and a further 120 min, respectively [24].

The digestion kinetics were described by means of a non-linear model in the following equation found by Goñi et al. [25]:

$$C = C_{\infty}(1 - e^{-kt}), \quad (1)$$

where C is the hydrolysis degree at each time, C∞ the maximum hydrolysis extent and k is the kinetic constant. The hydrolysis index (HI) was calculated as the relation (as percentage) between the area under the hydrolysis curve (AUC, 0–180 min) of each sample and the AUC of white bread as reference food. Previous research has shown HI to be a good predictor of glycemic response [25]. Last, expected glycemic index (eGI) was calculated using the equation proposed by Goñi et al. [25]:

$$\mathrm{eGI} = 39.71 + 0.549\mathrm{HI}. \quad (2)$$

Protein determination

Grass pea protein content was calculated by estimating nitrogen content [26].

In vitro protein digestion models

The simulation of human oral and gastric digestion of both unmodified and TG-modified grass pea flour was carried out following the procedure described in Giosafatto et al. [27].

At the end of the digestion experiment, 20 µL of each sample was analyzed by SDS-PAGE (4–20%).

SDS-PAGE

5 µL of sample buffer was added to aliquots of 20 µL of each sample and analyzed by 4–20% SDS-PAGE, as described by Laemmli [28]. Electrophoresis was performed at constant voltage (80 V for 2–3 h), and the proteins were stained with Coomassie Brilliant Blue R250. Bio-Rad Precision Protein Standards were used as molecular weight markers.

Image analysis

The SDS-PAGE gel images were acquired using Bio-Rad ChemDoc Imager. The image analysis was carried out using Image Lab software (Bio-Rad, version 5.2.1) following the procedure described in Giosafatto et al. [27].

Statistical analysis

All experimental results are reported as means and standard deviation of at least three independent experiments. One-way ANOVA with Duncan's multiple comparison test at the 95% confidence level ($p \leq 0.05$) were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) on all experimental data.

Results and discussion

TG-mediated modification of grass pea flour

To study the effect of protein structure on the biological properties of grass pea proteins, TG-mediated crosslinking assays were performed. It is worth to note that TG has been successfully exploited by our research group to modify several proteins of food interest. In particular, recently proteins from other legumes were subjected to TG treatment as reported in Romano et al. [17], who have modified, by means of the microbial enzyme, proteins from beans (*Phaseolus vulgaris*). On the other hand, Porta et al. [21, 29] have used TG to treat the proteins from bitter vetch (*Vicia ervilia)*, another legume used mainly for animal feeding, with the aim to prepare biopolymer materials with improved technological properties. In this study, we have performed some experiments that successfully demonstrated that also the proteins from grass pea, as from other legumes [14, 30, 31] act as effective substrate for TG. In particular, 100 µg of protein flour was incubated with increasing amount of Activa (5, 10 and 20 U/g of proteins, respectively) for 2 h at 37 °C. At the end of incubation, the TG assay was stopped by boiling the samples for 2 min. The extent of polymerization was evaluated by SDS-PAGE. It is worth to point out that the same assay was carried out on both denatured and undenatured proteins to verify the effect of protein denaturation on the extent of crosslinking. To this purpose, the flour was boiled for 15 min and, in this way, besides promoting the denaturation, we also mimicked the cooking process. As it is possible to see from Fig. 1, TG is able to catalyze the formation of high MW polymers with the concomitant decrease of the grass pea characteristic protein bands even using the lowest amount of TG (5 U/g). However, this effect is much more evident on the heat-treated samples. In fact, for obtaining the almost complete protein modification, 10 U/g of enzyme represents a sufficient amount for the denatured samples, whereas for the undenatured ones the same result is obtained only using 20 U/g of TG (Fig. 1). Nevertheless, the low MW bands, either from undenatured or denatured samples, seem quite resistant to act as TG substrate (Fig. 1). Under these experimental conditions, the grass pea proteins appear an effective substrate of TG and also more efficient than other legume proteins. Porta et al. [21] analyzing the effect of TG on proteins (undenatured) isolated from *Vicia ervilia* seeds have found that the lowest amount of enzyme able to lead to exhaustive polymerization is equal to 20 U/g; on the other hand phaseolin, main storage protein of the

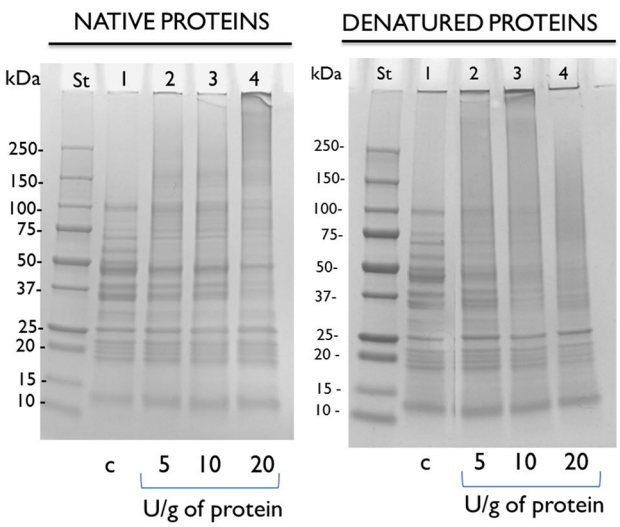

Fig. 1 TG-catalyzed reaction of grass pea flour proteins. Both native and undenatured flour proteins (100 µg) were incubated in the absence (lane 1, C) and presence of increasing amounts (lane 2–4) of Activa for 2 h at 37 °C. At the end of incubation, the samples were analyzed by SDS-PAGE (4–20%). St, Bio-Rad Precision Protein Standards were used as molecular weight markers. Further details are described in the text

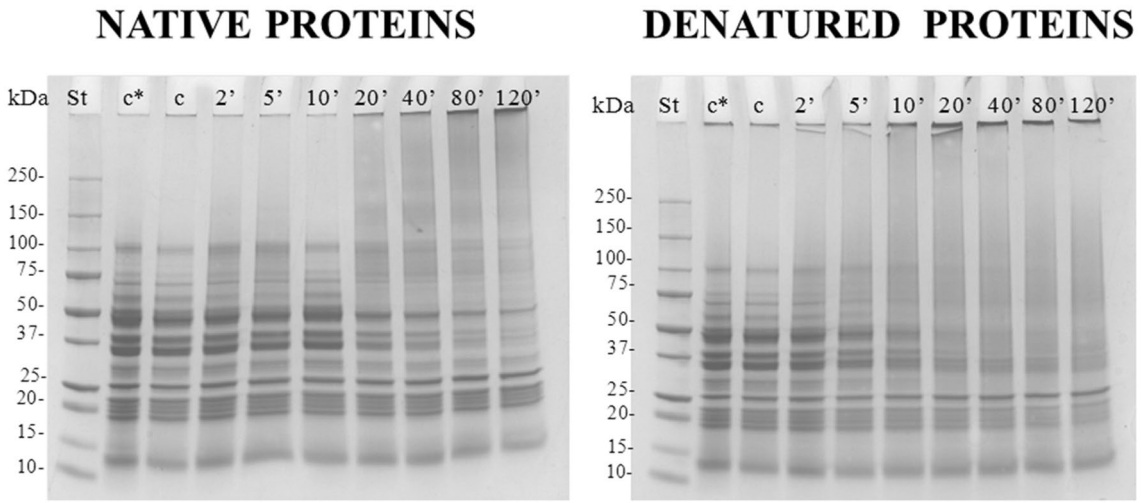

Fig. 2 TG-catalyzed reaction of grass pea flour proteins. Both native and undenatured flour proteins (100 µg) were incubated in the presence of 5U/g of Activa for different times at 37 °C. At the end of incubation, the samples were analyzed by SDS-PAGE (4–20%). c* and c are the controls representing the protein samples treated without Activa, not incubated and incubated for 2 h, respectively. St, Bio-Rad Precision Protein Standards were used as molecular weight markers. Further details are described in the text

seeds of *Phaseolus vulgaris*, can be modified after 2-h incubation using 15 U/g of microbial enzyme [14].

To prove that the polymerization is enzyme dependant, a time-course assay was also performed (Fig. 2). As it is possible to note from Fig. 2, the enzyme catalyzes the formation of intermolecular crosslinks among protein molecules more rapidly when the proteins are denatured. The 50-kDa protein band is still present at the end of incubation (120 min) when the proteins are not heat treated, while in the case of thermic denaturation already after 80 min such a band disappears together with other proteins possessing a MW ≥ 24 kDa. In addition, the formation of polymers that have a MW ≥ 250 kDa and of polymers unable to enter the gel is already evident after 10 min if the proteins are denatured. In the case of undenatured samples, the high MW polymers appear only over 20-min incubation.

Microstructure characteristics of flour

To study the microstructural changes arising as a result of the TG treatment, the microstructure of grass pea flour samples was investigated by means of SEM. Figure 3 shows representative SEM micrographs of grass pea flour samples: GP (Fig. 3a) and GP/TG (Fig. 3b).

GP samples (Fig. 3a) possess starch granules that appear swelled compared to the ones present in GP raw flour studied by Romano et al. [13] which contained oval and ellipsoid starch granules with heterogeneous sizes. The swelled aspect of starch granules in incubated GP samples is due to the partial hydration of their amorphous regions (partially gelatinized). On the top surface of starch granules, strands of protein bodies were also observed.

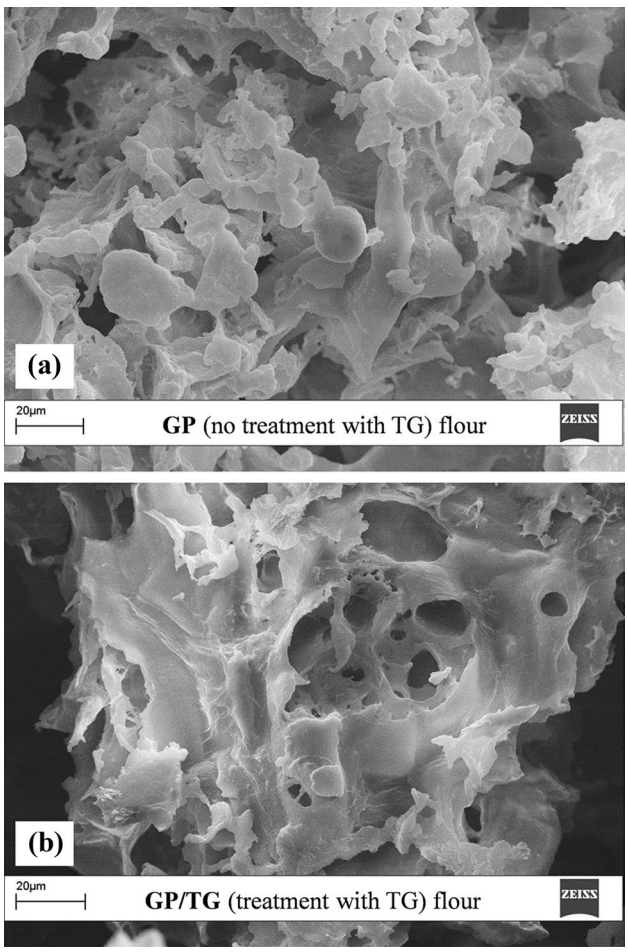

Fig. 3 Scanning electron micrographs (2000 K) of grass pea flour incubated for 2 h at 37 °C in the absence of TG (GP, panel a), and in the presence of TG (GP/TG, panel b)

As expected, GP/TG samples possessed a distinctly different structure (Fig. 3b) from raw flour [13] and GP samples (Fig. 3a). GP/TG samples showed in fact a more compact and homogeneous structure due, most probably, to the formation TG-mediated crosslinking that reinforces the protein–protein interactions. Similar microstructural observations were reported previously by Romano et al. [17] when TG was added to bean flour and by Bonet et al. [32] that studied the glucose oxidase effect on wheat flour dough at molecular level.

In vitro starch digestibility and expected glycemic index

The results regarding starch digestibility (in vitro) is depicted in Fig. 4. In vitro starch digestion was investigated by measuring the released glucose content during starch digestion and the hydrolysis curves of samples were compared with those performed by white bread used as control. All of the samples investigated showed a starch digestibility lower than white bread. The raw samples showed the lowest digestibility. The hydrolysis curves of GP flour showed a significantly ($p < 0.05$) increase upon 120 min with a more starch hydrolysis and digestibility than GP/TG. This increase could be due to the presence of swelled starch granules observed in Fig. 3a. The hydrolysis kinetics of GP/TG flour showed a similar hydrolysis trend, although the percentage of digested starch is lower at the plateau. This result could be explained because of the presence of TG-mediated protein network that could give rise to insoluble complexes also affecting in vitro digestibility of proteins [17].

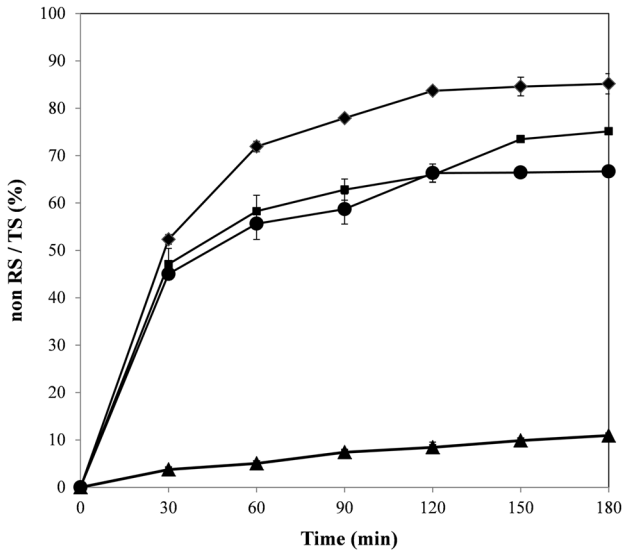

Fig. 4 Total starch hydrolysis rate of white bread (filled diamond) and grass pea flour: not incubated (filled triangle), incubated for 2 h at 37 °C in the absence of TG (filled square) and in the presence of TG (filled circle)

Starchy food can be classified according to their digestibility. Rapidly digestible starch (RDS) and slowly digestible starch (SDS) of different starches are shown in Table 1. RDS is rapidly and completely digested in the small intestine and is associated with more rapid elevation of postprandial plasma glucose, whereas SDS is more slowly digested in the small intestine and is generally the most desirable form of dietary starch [23, 33]. The RDS content was in the range of 1.5–18.7%, while the SDS contents ranged from 3.3 ± 0.4% of raw flour to 26.1 ± 0.6% of GP. In particular, the GP/TG samples showed significantly ($p < 0.05$) lower RDS, SDS (Table 1) in comparison with GP samples. Certain indigestible polymers and some associated non-fibrous compounds may, in fact, reduce the rate of starch digestion in vitro and in vivo, resulting in low metabolic responses [34].

The expected glycemic index (eGI) for different samples is shown in Table 1; eGI for the flour samples was in order GP>GP/TG>raw. The eGI results differed significantly ($p < 0.05$) varying between 45.03 of raw flour and 85.58% of grass pea flour without TG (Table 1). The eGI was correlated with the parameters of the starch fractions, including RDS and RS. In particular, RDS is found to be a positive and main contributing factor to the eGI. Higher percentages of RDS in starch are usually related to a higher degree of eGI [35, 36], while the RS content had an inverse relationship with eGI [24]. eGI influences the nutritional quality of foods and the benefits of a low GI food in reducing insulin demand, improving satiety, improving blood glucose control with diabetic people, reducing blood lipid level and increasing colonic fermentation which are well documented [37]. The protein network formed reduces the rate of grass pea flour starch digestion, being the glycemic index of grass pea flour modified by TG lower than the non-treated one (GP).

Considering the in vitro digestibility results of TG samples, the latter might be a potential ingredient in the formulation of products for diabetics and weight management, and could lead to the formulation of novel foods characterized by the slow release of glucose, that is to say low glycemic index and prevention of fasting hypoglycemia.

Table 1 Effect of TG on starch nutritional fractions (RDS, rapidly digestible starch, and SDS, slowly digestible starch) and expected glycemic index (eGI) of the analyzed samples. Each value is expressed as mean ± S.D

Grass pea flour	RDS (%)	SDS (%)	eGI (%)
Raw	1.46 ± 0.21^{a}	3.29 ± 0.42^{a}	45.03 ± 0.18^{a}
GP	18.65 ± 1.32^{c}	26.10 ± 0.61^{c}	85.58 ± 0.37^{c}
GP/TG	16.67 ± 0.09^{b}	24.55 ± 0.70^{b}	82.97 ± 0.53^{b}

[a–c]Means within the same column with different letters are significantly different ($p < 0.05$; Duncan test)

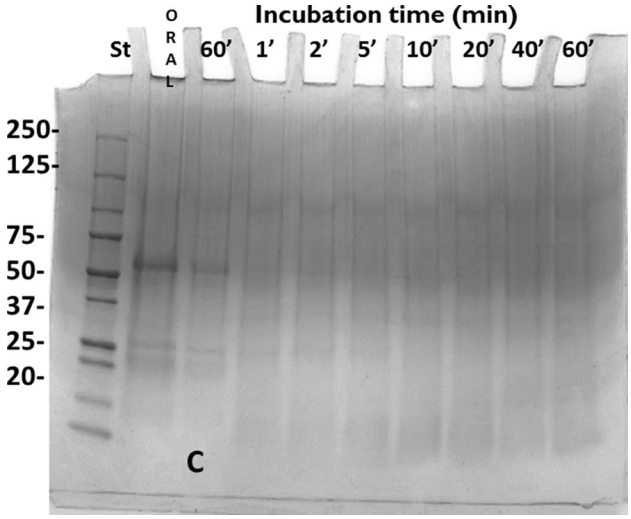

Fig. 5 Digestibility of grass pea flour proteins. TG-treated samples were subjected to oral and gastric digestion following the conditions found in the adult human. At the end of incubation, the samples were analyzed by SDS-PAGE (4–20%). Lane "c" corresponds to the samples before the digestion. St, Bio-Rad Precision Protein Standards were used as molecular weight markers. Further details are described in the text

Protein digestibility

In this work, we have also studied the digestibility of grass pea flour proteins following TG treatment, to study the effect of food structure on the human gut. To this aim, the TG (Activa, 20 U/g)-modified sample was subjected to in vitro oral and gastric digestion carried out under physiological conditions as described in Giosafatto et al. [27] and the products are analyzed by SDS-PAGE. The results reported in Fig. 5 demonstrated that TG did not influence the digestibility of grass pea flour proteins. In fact, the crosslinked polymers (with a MW ≥ 250 kDa) as the unmodified proteins [13] were still easily and gradually digested upon incubation with pepsin. In fact, densitometry analysis shows that only about 20% of TG-modified forms were present after 20-min gastric digestion in comparison with control (Fig. 5). At the end of incubation with pepsin, only 13% of high MW polymers are still detectable. These results are very interesting since some previous papers showed that food processing influences the protein digestion [38, 39] and in particular, TG-mediated crosslinked protein forms appear very stable and more resistant to the hydrolysis catalyzed by digestive enzymes. In fact, Giosafatto et al. [13] demonstrated that ovalbumin polymers obtained by means of TG persisted even through duodenal digestion suggesting that the TG-induced crosslinking of the egg protein affects the rate of digestion. Similar results were also observed by Tang et al. [40] and Monogioudi et al. [41] that found the covalent crosslinking of soy as well as β-casein decreased the in vitro digestibility especially that observed for pepsin digestion. Nevertheless, cucurbitin protein from pumpkin oil cake crosslinked by TG was still prone to be digested by gastrointestinal enzymes and the obtained hydrolysates still maintained their bioactive potential [42]. Based on our results, it is possible to assess that TG was able to modify grass pea flour proteins providing a novel flour ingredient which might be used to obtain food highly digestible and with a low glycemic index. Nowadays, the demand of "easy to use" foods is increasing in western countries; thus the results reported in the present paper could represent a basic study to develop sustainable novel foods with desired characteristics for different groups of consumers, such as athletes, diabetics or common people that do care about a healthy diet.

Conclusions

Grass pea flour proteins either or not heat treated act as effective TG substrate, even though the heat processing markedly improves the capability of these proteins to be modified by the microbial enzyme. The TG-crosslinked proteins were easily digested in vitro and possess nutritional properties that make grass pea an inexpensive legume suitable for feeding a large spectrum of population.

Acknowledgements The authors thank Mrs Maria Fenderico for their technical collaboration.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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